J. Biochem. 126, 715-721 (1999)

Specific Efflux of Glutathione from the Basolateral Membrane Domain in Polarized MDCK Cells during Ricin-Induced Apoptosis

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Received May 27, 1999; accepted July 29, 1999

Although the depletion of reduced glutathione (GSH) has been observed in a variety of apoptotic systems, little is known about the mechanism of GSH depletion. In this study we used polarized MDCK cells to study the GSH flux during ricin-induced apoptosis. Here we report that the specific accumulation of GSH occurred in the basolateral medium during ricin treatment with similar kinetics to in apoptotic changes such as an increase in caspase-3 like activity and DNA fragmentation, while there was no significant increase in the GSH level in apical medium. These results suggest that GSH efflux occurred through a GSH-specific channel or transporter located in the basolateral membrane domain of polarized MDCK cells undergoing apoptosis. Treatment with other protein toxins such as modeccin, *Pseudomonas* toxin, and diphtheria toxin, which can induce apoptotic cell death, also resulted in selective GSH efflux from the basolateral side. Thus, GSH efflux through a specific transporter may be a common step of apoptosis induced by these toxins, while these toxins have different intoxication mechanisms leading to protein synthesis inhibition. Pretreatment of cells with Z-Asp-CH_2-DCB, a caspase family inhibitor, inhibited ricin-induced basolateral GSH efflux as well as DNA fragmentation, suggesting that the activation of caspases, *i.e.* those that are inhibited by Z-Asp-CH_2-DCB, is implicated in the opening of the GSH transporter.

Key words: apoptosis, caspases, GSH efflux, ricin, polarized MDCK cell.

Ricin, a plant protein toxin, is internalized into mammalian cells through receptor-mediated endocytosis. Following intracellular vesicle trafficking through the Golgi apparatus, ricin or its enzymatically active component (A-chain) is eventually translocated into the cytosol to reach its target, *i.e.*, the 28S RNA of the 60S ribosomal subunit (*1-3*). Recent studies have demonstrated that ricin and other protein toxins, such as modeccin, diphtheria toxin, and *Pseudomonas* toxin, induce apoptotic cell death (*4-8*). In cells undergoing apoptosis, there are characteristic morphological and biochemical changes, which include cytoplasmic shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation and the appearance of apoptotic bodies. In fact, some of these characteristic apoptotic features have been observed in ricin-treated cells (*4, 6, 8*).

Increasing evidence has demonstrated that the members of a unique family of cysteine proteases designated as caspases play critical roles in the control of apoptotic cell death (*9, 10*). The interleukin-1β-converting enzyme (ICE, *i.e.*, caspase-1) was the first identified member of the caspase family, and is the mammalian homologue of CED-3, the product of a gene essential for programmed cell death in the nematode, *Caenorhabditis elegans* (*11*). Recent studies suggested that multiple caspases are involved in the progress of apoptosis in mammalian cells (*12, 13*).

In addition to the specific proteolytic cleavage events in the apoptotic signaling pathway, depletion of intracellular glutathione, the most abundant intracellular thiol-containing tripeptide (γ-glutamylcysteinylglycine), was recently found to occur in several different apoptotic systems, and it has been suggested that the depletion of glutathione is a relatively early event in the commitment to apoptosis (*14-17*). For example, thymocytes undergoing apoptosis after exposure to glucocorticoid or DNA damaging agents lose intracellular glutathione, with similar kinetics to in internucleosomal chromatin fragmentation (*16*). Similarly, we previously reported that ricin-induced apoptosis of U937 cells is associated with glutathione depletion; glutathione is extruded in the reduced form (GSH) during the apoptotic processes before any plasma membrane leakage, indicating that GSH loss in ricin-induced apoptosis is not a simple consequence of oxidative stress (*18*). However, how intracellular glutathione is depleted remains to be clarified. To further understand the mechanism of GSH depletion, the glutathione flux in polarized MDCK cells exposed to ricin was investigated. As GSH efflux occurred selectively from basolateral membrane domains in polarized MDCK cells

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Abbreviations: DTNB, 5,5'-dithio-bis (2-nitrobenzoic acid); BSA, bovine serum albumin; LDH, lactate dehydrogenase; Z-Asp-CH_2-DCB, carbobenzoxy-Asp-1-yl-[(2,6-dichlorobenzoyl)oxy]methane; Ac-DEVD-CHO, acetyl-Asp-Glu-Val-Asp-aldehyde; Ac-YVAD-CHO, acetyl-Tyr-Val-Ala-Asp-aldehyde; Ac-VEID-CHO, acetyl-Val-Glu-Ile-Asp-aldehyde; MCA, 4-methyl-coumaryl-7-amide; GSH, glutathione; GSSG, glutathione disulfide; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; ICE, interleukin-1β-converting enzyme; PBS, phosphate-buffered saline.

after exposure to ricin, the selective opening of a GSH-specific membrane channel or transporter is proposed to be responsible.

MATERIALS AND METHODS

Materials—Ricin was obtained from Sigma Chemical (St. Luis, MO). Ricin was also isolated from small castor beans as described by Mise *et al.* (*19*). Diphtheria toxin and *Pseudomonas* toxin were purchased from the Swiss Serum and Vaccine Institute (Berne, Switzerland). Modeccin was obtained from Inland Laboratories (Austin, TX). The fluorescent tetrapeptide substrates of proteases (Ac-DEVD-MCA and Ac-YVAD-MCA) and caspase related protease inhibitors (Ac-DEVD-CHO, Ac-YVAD-CHO, Ac-VEID-CHO, and Z-Asp-CH_2-DCB) were obtained from Peptide Institute, Osaka, Japan. 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) was purchased from Nacalai Tesque, Kyoto. [^{3}H]Leucine (60 Ci/mmol) was obtained from NEN Research Products (Boston, MA).

Cell Culture—MDCK (Madin-Darby canine kidney) cells were maintained in culture in α-minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS), 10 μg each of adenosine, guanosine, cytidine, and thymidine per ml of medium, penicillin (100 μg/ml), and streptomycin (100 μg/ml), as described previously (*20*). For the experiments on polarized MDCK cells, cells were seeded on polycarbonate filters (Costar Transwell; pore size 0.4 μm, diameter 12 mm) at a density of 3×10^5 cell/filter and used 2 days later. The filters were rinsed twice with α-MEM, and then the experiments were performed with 0.5 and 1.5 ml of serum-free α-MEM containing 35 μM BSA in apical and basal chambers, respectively. Treatment of cells with toxins was always performed in apical chambers. After the indicated periods of time, the medium was collected from both chambers and subjected to glutathione assaying.

Glutathione Measurement—Glutathione was determined by the recycling assay based on the reduction of 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) with glutathione reductase and NADPH (*21*). The sample preparation and assay procedures were described elsewhere (*18*).

Measurement of Protein Synthesis Inhibition—Cells were inoculated at a density of 1×10^5 cells/well in 0.2 ml of medium in 48-well plates and used for experiments one day later. After 3-h treatment with each toxin in serum-free α-MEM containing 35 μM BSA at 37°C, the cells were incubated with 0.5 μCi/ml [^{3}H]leucine for 45 min at 37°C in leucine-free medium. The incorporation of [^{3}H]leucine into perchloric acid/phosphotungstic acid-insoluble materials was determined as described previously (*20*). The results were expressed as a percentage of the incorporation in control cells incubated without toxin but otherwise treated in the same way. For the time course analysis, cells were incubated with ricin (10 ng/ml) for the indicated periods of time at 37°C, and then the rate of protein synthesis was measured by incubating the cells with leucine-free medium containing 10 μCi/ml [^{3}H]leucine for 10 min at 37°C as described above.

Peptide Cleavage Assay—Cell monolayers in dishes (35 mm) (1×10^6 cells/dish) were incubated with 10 ng/ml of ricin in α-MEM containing 35 μM BSA at 37°C for the indicated periods of time, and then washed twice with PBS and suspended in 200 μl of extraction buffer (10 mM HEPES/KOH buffer, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 1 mM PMSF) as described (*12*). After repeated freezing and thawing, cell debris was removed by centrifugation at $13,000\times g$ at 4°C for 20 min. The supernatants were incubated with 10 μM fluorescent substrate at 37°C for 10 min, and then peptide cleavage was analyzed with excitation at 380 nm and emission at 460 nm. The specific inhibitor for caspase-1 (Ac-YVAD-CHO) or caspase-3 (Ac-DEVD-CHO) was added to the reaction mixture at a concentration of 4 μM. Specific caspase-like activities were determined by subtracting the values obtained in the presence of each inhibitor.

Quantitation of DNA Fragmentation—Cell monolayers in dishes (35 mm) (1×10^6 cells/dish) were treated with 10 ng/ml of ricin in α-MEM containing 35 μM BSA at 37°C for the indicated periods of time. After removal of the medium, the cells were lysed in 1 ml of cold lysis buffer containing 0.5% Triton X-100, 10 mM Tris, 20 mM EDTA, pH 8.0. Samples were subsequently centrifuged for 28 min at $13,000\times g$ to separate DNA fragments (supernatant) from intact DNA (pellet). The DNA contents of the supernatant and pellet fractions was determined using the diphenylamine reagent (*22*).

Nuclear Staining—MDCK cells grown on glass coverslips were incubated with ricin at 37°C in α-MEM containing 35 μM BSA. The cells were washed with PBS, and then fixed with 1% glutaraldehyde for 30 min at room temperature. Then the cells were stained with Hoechst 33258 (0.15 mM) for 10 min at room temperature, and observed by fluorescence microscopy (Olympus IMT-2).

Cytolytic Activity—The cytolytic activity of toxins was measured by means of the lactate dehydrogenase (LDH) release assay, in which LDH released from lysed cells was determined by measurement of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) reduction, as described previously (*18*). In brief, 3×10^4 cells/well in a 96-well plate in α-MEM containing 35 μM bovine serum albumin (BSA) were treated with varying concentrations of toxin for 24 h at 37°C. Following centrifugation of the plate ($300\times g$, 10 min), 50 μl of the supernatant in each well was subjected to the LDH assay.

RESULTS

Kinetics of Ricin-Induced Protein Synthesis Inhibition and Apoptotic Events in MDCK Cells—Since the dose-response curve showed that 10 ng/ml of ricin caused almost complete inhibition of protein synthesis in MDCK cells, this concentration of ricin was used for further studies (inset in Fig. 1). The lag period before the onset of inhibition of protein synthesis by ricin after its binding to cell surface receptors is generally assumed to correspond to the intracellular events that lead to the translocation of ricin into the cytosol. As shown in Fig. 1, ricin (10 ng/ml) required about 3 h for significant inhibition of protein synthesis in MDCK cells. We previously reported that ricin, aside from its protein synthesis inhibitory activity, can also cause a nuclear morphological change, DNA fragmentation, an increase in caspase-like activities, and eventual cytolysis in U937 cells. All of these changes are characteristic of cells undergoing apoptosis (*23*). To examine the temporal relationship between ricin-induced pro-

tein synthesis inhibition and these apoptotic changes, we carried out time course studies on DNA fragmentation, activation of caspase-like activities, and cell lysis in ricin-treated MDCK cells. As shown in Fig. 2A, treatment of MDCK cells with ricin (10 ng/ml) caused DNA fragmentation, and this process started at 4 h, *i.e.* just after protein synthesis had largely been inhibited. During ricin-treatment, gradual rounding of adherent MDCK cells was observed, and almost all the rounded cell had accompanying apoptotic nuclear morphological changes and eventually became detached from the plate (Fig. 3). Consistent with the results for U937 cells (*23*), an increase in caspase-3 like activity was observed, whereas no significant caspase-1 like activity was detected during ricin-treatment (Fig. 2B). The kinetics of the activation of caspase-3 like activity were similar to those of DNA fragmentation, suggesting these apoptotic events are closely related in terms of the time schedule. On the other hand, ricin-mediated cytolysis, as measured as LDH release, occurred much later than DNA fragmentation, and at least a 6 h lag period preceded the onset of LDH release (Fig. 2C). Taken together, our results suggest that protein synthesis inhibition caused by ricin may be recognized by the target cells as a cytotoxic stress, which triggers the apoptotic process leading to eventual cytolysis.

Glutathione Efflux in Ricin-Treated Polarized MDCK Cells—We previously reported that glutathione (GSH) loss is observed in ricin-treated U937 cells at a relatively early step leading to apoptosis, before any plasma membrane leakage, suggesting that specific extrusion has taken place. To gain an insight into the mechanism responsible for apoptotic GSH extrusion, we investigated the effect of ricin on the flux of glutathione in polarized MDCK cells. As shown in Fig. 4, a time-dependent increase in the GSH level in the basolateral medium was observed after exposure to ricin, with similar kinetics to in the appearance of caspase-3 like activity (Fig. 2A), whereas there was no significant accumulation of GSH in the apical medium. When ricin was added on the basolateral side, basically similar results were obtained (data not shown). The initial level of intracellular GSH was 13.07 ± 0.7 nmol/10^6 cells, and no significant change in the combined amount of intra- and extracellular GSH was observed during 8 h incubation. More than 95% of the glutathione detected in the medium was in the reduced form and no significant increase in the GSSG level was observed (data not shown). Furthermore, leakage of cytosolic LDH even into the basolateral medium was not observed at least during 8 h ricin-treatment (data not shown). These results suggest that selective efflux of GSH occurs through specific transporters or channels located in the basolateral plasma membrane domain in polarized MDCK cells during ricin-induced apoptosis.

Several GSH-specific plasma membrane transporters

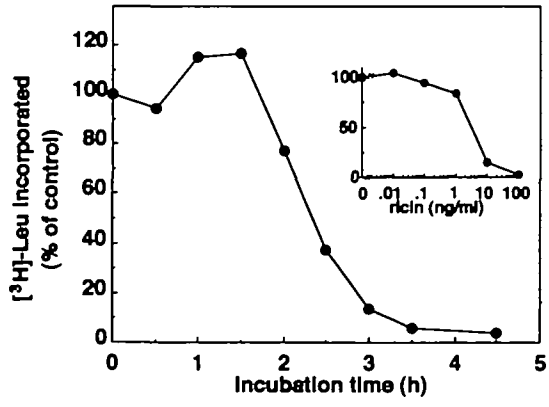

Fig. 1. **Cytotoxicity of ricin in MDCK cells.** The cytotoxicity of ricin was measured as the inhibition of [³H]leucine incorporation. Cells grown in 48-well plates (1×10^5 cells/well) were incubated with varying concentrations of ricin in α-MEM containing 35 μM BSA for 3 h at 37°C (inset). Cells were labeled with [³H]leucine (0.5 μCi/ml) for 45 min in leucine-free medium for the measurement of protein synthesis. The time course of protein synthesis of MDCK cells exposed to 10 ng/ml of ricin was determined at the indicated times as described under "MATERIALS AND METHODS." Each point represents the average of duplicate measurements.

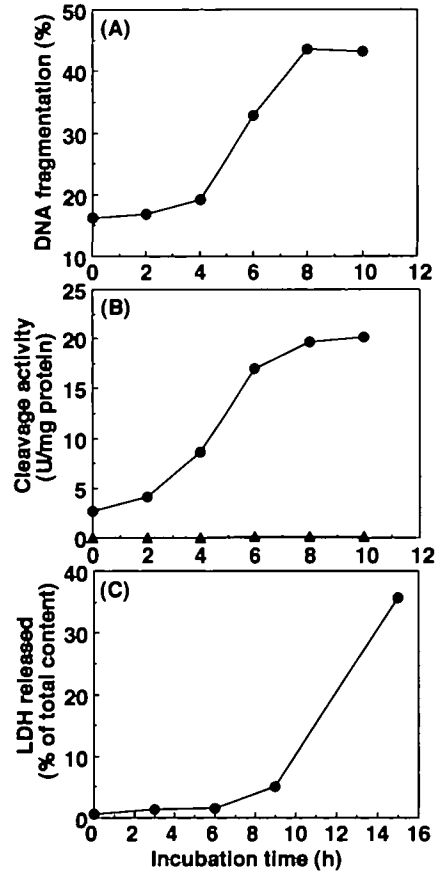

Fig. 2. **Time course analysis of DNA fragmentation (A), activation of caspase-like protease activities (B), and cytolysis (C) in ricin-treated MDCK cells.** (A) Cells (1×10^6 cells/dish) were incubated with 10 ng/ml of ricin in α-MEM containing 35 μM BSA for the indicated periods of time at 37°C. DNA fragmentation in ricin-treated cells was assayed with diphenylamine as described under "MATERIALS AND METHODS." (B) After the preparation of cytosolic extracts from ricin-treated MDCK cells (1×10^6 cells/dish), caspase-1- (▲) and caspase-3-like (●) activities in the lysates were determined using the fluorescent substrates, Ac-YVAD-MCA and Ac-DEVD-MCA, respectively. (C) Cells in 96-well plates (3×10^4 cells/well) were incubated with 10 ng/ml of ricin in α-MEM containing 35 μM BSA for the indicated periods of time at 37°C, and then subjected to the LDH release assay as described under "MATERIALS AND METHODS." All measurements were performed in duplicate. The differences between the values in duplicate experiments were within 10%.

have been described (*24-26*). Rat hepatocytes have been shown to have sinusoidal GSH transporters located in the basolateral membrane, which governs GSH efflux into blood, and this type of transporter is known to be inhibited by L-methionine or cystathionine (*24, 25*). Thus, we examined whether or not L-methionine and cystathionine inhibit the efflux of GSH from ricin-treated polarized MDCK cells. As shown in Fig. 5A, these reagents had almost no effect on ricin-induced GSH efflux into the basolateral medium, suggesting that the GSH transporter, which is probably located in the basolateral membrane in MDCK cells, exhibits some difference with respect to its inhibitor sensitivity compared to the sinusoidal membrane GSH transporter in rat hepatocytes. Furthermore, these inhibitors had no effect on ricin-induced DNA fragmentation, whereas exogenous GSH showed a partial protective effect against ricin-induced DNA fragmentation (Fig. 5B).

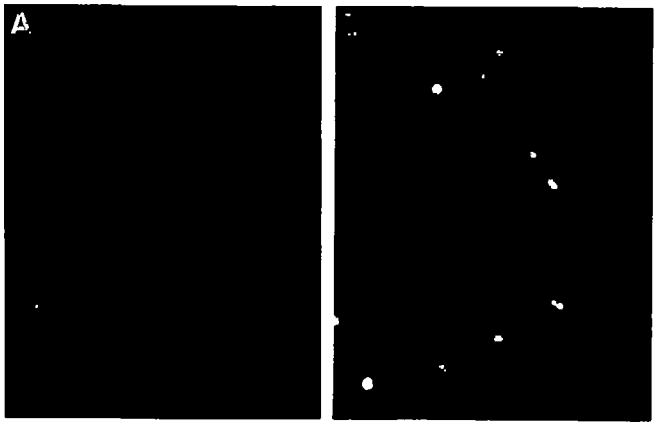

Fig. 3. **Ricin-induced nuclear morphological changes in MDCK cells.** Cells grown on glass coverslips were incubated without (A) or with (B) 10 ng/ml of ricin for 10 h at 37°C. After treatment, the cells were fixed and stained with Hoechst 33258 (0.15 mM) for 10 min, and then observed under a fluorescence microscope.

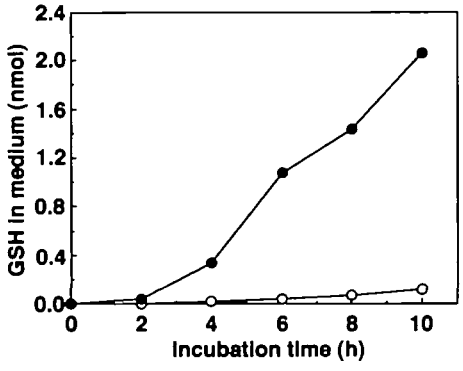

Fig. 4. **Time course of GSH efflux from ricin-treated polarized MDCK cells.** To confluent cells grown on a filter, 10 ng/ml of ricin was added on the apical side, and then the cells were incubated in α-MEM containing 35 μM BSA for the indicated periods of time at 37°C. Then the medium was collected from both sides and subjected to the glutathione assay as described under "MATERIALS AND METHODS." Each point represents the total GSH (nmol) detected in apical (0.5 ml) (○) and basolateral (1.5 ml) (●) medium. Each point represents the average of duplicate measurements. The experiment was repeated several times and basically similar results were obtained.

It has been shown that Z-Asp-CH_2-DCB, a caspase family protease inhibitor, completely inhibits the generation of caspase-3 and -6 like activities in ricin-treated U937 cells as well as the inhibition of all features of apoptosis, such as nuclear morphological changes, DNA fragmentation and eventual cell lysis (*23*). To investigate the relationship between GSH efflux and the activation of caspase-related proteases that are believed to initiate apoptotic chromatin condensation, polarized MDCK cells were preincubated with Z-Asp-CH_2-DCB. Consistent with a previous report, this inhibitor was found to inhibit both ricin-induced GSH efflux and DNA fragmentation. The generation of caspase-3-like activity was also prevented by the inhibitor (Table I). These results indicate that the GSH efflux caused by ricin is dependent on the activation of caspase family proteases. Consistent with our previous results (*18*),

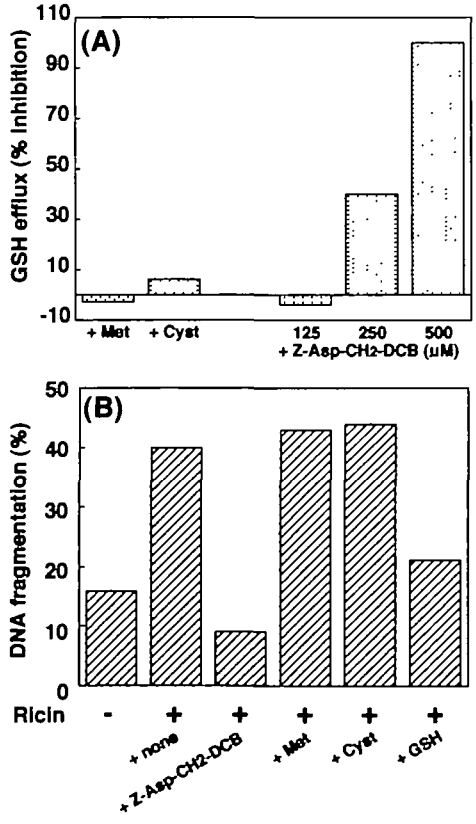

Fig. 5. **Effects of various reagents on the ricin-induced GSH efflux from the basolateral side and DNA fragmentation in polarized MDCK cells.** (A) Filter-grown cells were preincubated with methionine (10 mM), cystathionine (10 mM), or the indicated concentration of Z-Asp-CH_2-DCB for 1 h in α-MEM containing 35 μM BSA at 37°C. After the addition of ricin (10 ng/ml) on the apical side, cells were incubated for another 8 h at 37°C. Then the GSH levels on the basolateral side were determined as described in the legend to Fig. 4. The data were expressed as percent inhibition against the level of GSH efflux induced by ricin alone. (B) Cells (1×10^6 cells/dish) were preincubated with methionine (10 mM), cystathionine (10 mM), GSH (10 mM), or Z-Asp-CH_2-DCB (500 μM) for 1 h in α-MEM containing 35 μM BSA at 37°C, followed by the addition of ricin (10 ng/ml). After another 8 h incubation at 37°C, the cells were subjected to the DNA fragmentation assay as described under "MATERIALS AND METHODS." All measurements were performed in duplicate. The differences between the values in duplicate experiments were within 10%.

TABLE I. **Effect of Z-Asp-CH_2-DCB on the generation of caspase-3 like activity in ricin-treated MDCK cells.**

	Caspase-3 like activity (U/mg cell protein)
None	3.5±0.2
+Ricin	24.2±0.5
+Ricin and Z-Asp-CH_2-DCB (500 μM)	0.4±0.0

Cells were preincubated with or without 500 μM Z-Asp-CH_2-DCB for 1 h in α-MEM containing 35 μM BSA at 37°C. After the addition of ricin (10 ng/ml), the cells were incubated for another 8 h at 37°C. The caspase-3 like activity in the cell extract was determined as described in the legend to Fig. 2. The results are the means±SD of triplicate determinations.

tetrapeptide caspase-related inhibitors, such as Ac-DEVD-CHO, Ac-YVAD-CHO, and Ac-VEID-CHO, had almost no effect on basolateral GSH efflux in ricin-treated polarized MDCK cells even at 500 μM, and none of them could prevent ricin-induced DNA fragmentation (data not shown). However, these results do not necessarily mean that caspase-1, -3, and -6 are not involved in the GSH efflux, since there is a possibility that the inability of these inhibitors may be due to their poor membrane permeability in MDCK cells. Further studies are required for identification of the caspases responsible for apoptotic GSH efflux.

Effects of Modeccin, Pseudomonas Toxin, and Diphtheria Toxin on Glutathione Flux in Polarized MDCK Cells— Other protein toxins, such as modeccin, *Pseudomanas* toxin, and diphtheria toxin, are also known to induce apoptotic cell death (*4-8*). To ascertain whether or not the selective GSH efflux from the basolateral side is specific to ricin-induced apoptosis, we measured the GSH levels in basolateral and apical media of polarized MDCK cells after treatment with modeccin, *Pseudomanas* toxin, and diphtheria toxin. Before the GSH flux experiments, we checked the protein synthesis inhibitory activities and cytolytic activities of these toxins as to MDCK cells. The extents of protein synthesis inhibition (Fig. 6A) by these toxins paralleled their cytolytic activities (Fig. 6B), and the concentrations of toxins required for strong cytolysis were found to be: ricin, 10 ng/ml; modeccin, 1,000 ng/ml; diphtheria toxin, 100 ng/ml; and *Pseudomonas* toxin, 10,000 ng/ml, and these concentrations were used in GSH flux experiments. As shown in Fig. 6C, predominant accumulation of GSH in the basolateral medium was observed for polarized MDCK cells treated with modeccin, *Pseudomonas* toxin, and diphtheria toxin, similar to in the case of ricin. Therefore, these results suggest that GSH efflux from the basolateral membrane domain is a common step in the apoptotic pathways of these protein toxins.

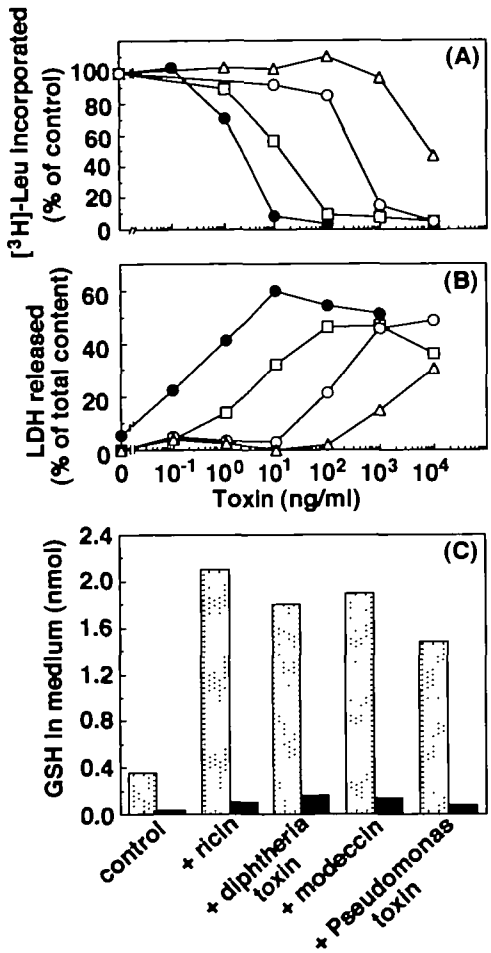

Fig. 6. **Protein synthesis inhibitory (A) and cytolytic (B) activities of ricin (●), modeccin (○), *Pseudomonas* toxin (△), and diphtheria toxin (□), and their effects on GSH flux in polarized MDCK cells.** (A) Cells grown in 48-well plates (1×10^5 cells/well) were incubated with varying concentrations of each toxin in α-MEM containing 35 μM BSA for 3 h at 37°C, and then protein synthesis was measured as the incorporation of [^{3}H]leucine, as described in the legend to Fig. 1. (B) Cells in 96-well plates (3×10^4 cells/well) were incubated with varying concentrations of each toxin in α-MEM containing 35 μM BSA for 24 h at 37°C, and then subjected to the LDH release assay as described under "MATERIALS AND METHODS." (C) To filter-grown MDCK cells, ricin (10 ng/ml), modeccin (1,000 ng/ml), diphtheria toxin (100 ng/ml), or *Pseudomonas* toxin (10,000 ng/ml) was added on the apical side, and then the cells were incubated in α-MEM containing 35 μM BSA for 8 h at 37°C. Then the apical (▨) and basolateral (▤) media were collected from the chambers and subjected to the glutathione assay as described in the legend to Fig. 4. All measurements were performed in duplicate. The results for C are averages of duplicate determinations and are representative of three separate experiments. The differences between the values in duplicate experiments were within 10%.

DISCUSSION

Glutathione is the most abundant non-protein thiol-containing small molecule in mammalian cells. It plays an important role in cellular defense against oxidative stress by inactivating reactive oxygen species (ROS) or regulates several aspects of cellular metabolism. Depletion of reduced glutathione (GSH) has recently been reported to precede the onset of apoptotic cell death induced by various agents (*15, 17, 18, 27*), or to increase the sensitivity to apoptotic agents (*28, 29*), and it has been suggested that depletion of GSH is a relatively early event in the commitment to apoptosis (*27*). In addition, inhibitory or delaying effects of several antioxidants have been reported in many different forms of apoptosis (*18, 30, 31*). However, several recent reports indicated that ROS are not necessarily required for apoptosis, since cells cultured under near-anaerobic conditions, where ROS are unlikely to be produced, can still undergo apoptosis (*32, 33*). Therefore, it seems more likely that severe depletion of GSH leads to a decrease in the cellular reducing capacity and thereby

induces ROS-independent oxidative stress.

In the present study, we demonstrated that GSH efflux occurred selectively from basolateral membrane domains in polarized MDCK cells undergoing ricin-induced apoptosis, whereas no significant extrusion of GSH onto the apical side was observed (Fig. 4). These results suggest that the selective opening of a GSH-specific transporter located in the basolateral membrane domain of MDCK cells may be responsible for the GSH efflux during apoptosis. Consistent with our results, it was recently reported that GSH was directly exported from Jurkat T lymphocytes undergoing apoptosis after exposure to anti-Fas/APO-1 antibodies rather than depleted by oxidation (*17*). Regarding GSH-specific transporters or channels, Ghibelli *et al.* reported that U937 and HepG2 cells are protected from puromycin-induced apoptosis as a consequence of the inhibition of GSH efflux by two specific inhibitors of channel-mediated GSH extrusion, methionine and cystathionine (*34*). From these results, they concluded that apoptotic GSH extrusion occurs through specific transporters or carriers, and that it is required for commitment to apoptosis. Since ricin-induced apoptotic GSH efflux in MDCK cells was unaffected by cystathionine or methionine, in the case of MDCK cells, a different type of GSH-specific transporter might be responsible for the ricin-induced apoptosis.

Interestingly, ricin-induced GSH efflux was inhibited in a dose-dependent manner by the caspase family protease inhibitor, Z-Asp-CH_2-DCB. Pretreatment of MDCK cells with 500 μM Z-Asp-CH_2-DCB brought about almost complete inhibition of GSH efflux, and apoptotic DNA fragmentation was also fully blocked (Fig. 5A). These results clearly indicate that GSH efflux induced by ricin is dependent on the activation of caspase family proteases. Although Z-Asp-CH_2-DCB-sensitive caspases, which may govern GSH-specific transporters, remain to be identified, the notion described above may be supported by the finding that the efflux of GSH from Jurkat cells exposed to anti-Fas/APO-1 antibodies was inhibited by Z-Val-Ala-Asp-chloromethylketone, an inhibitor of ICE family proteases (*17*). Thus, one can speculate that oxygen-independent modification of intracellular redox states by GSH efflux *via* a specific GSH transporter, which is probably activated by caspase family proteases, may be a common signaling pathway leading to apoptotic cell death.

Although the exact physiological meaning of selective GSH efflux from the basolateral side is unknown, several possibilities can be imagined. First, the basolateral localization of the GSH transporter responsible for apoptosis may be a simple consequence of the fact that the apical and basolateral domains of polarized MDCK cells are functionally and compositionally distinct (*35*). Second, it is generally accepted that the segregation of the plasma membrane in epithelial cells into apical and basolateral domains is essential for their vectorial function *in vivo* (*36*). If the apoptotic process occurs in the epithelium lining a cavity, GSH released from the apical side may be lost in the lumen without having any significant physiological effect. In contrast, basolaterally exported GSH may be of some advantage for normal cells adjacent to an apoptotic neighbor. Thiol compounds including 2-mercaptoethanol and GSH are well known to enhance the growth and function of lymphoid cells (*37, 38*). Therefore, it is possible that basolaterally released GSH molecules are utilized by phagocytic cells for the recognition and phagocytosis of the apoptotic cells.

Although additional biochemical analysis is necessary to elucidate the mechanism of GSH efflux during ricin-induced apoptosis, our results clearly indicate the presence of a specific GSH transporter responsible for the apoptotic process, and extrusion of GSH through such a transporter is one of the early steps leading to apoptotic cell death. The identification and characterization of the apoptotic-related GSH transporter may contribute to further understanding of ricin-induced apoptotic cell death.

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